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Streptococcus cristatus attenuates *Fusobacterium nucleatum*-induced interleukin-8 expression in oral epithelial cells

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Background and Objective: Oral epithelial cells may be invaded by a polymicrobial intracellular flora, including pathogens together with commensals. Various oral pathogens can induce the production of interleukin-8, a potent neutrophil chemotractant, in oral epithelial cells. Evidence from the gut suggests that commensal species may modulate inflammatory responses to pathogens. The aim of this study was to examine the interleukin-8 responses of oral epithelial cells to an oral pro-inflammatory species, *Fusobacterium nucleatum*, in combination with an oral commensal, *Streptococcus cristatus*.

Material and methods: KB, TERT-2, TR146 and SCC15 cells were cocultured with *F. nucleatum* and *S. cristatus*, either alone or in combination, at 37°C in 5% CO_2 under various conditions. The mRNA expression of interleukin-8 was analyzed by reverse transcription–polymerase chain reaction and protein secretion was measured by enzyme-linked immunosorbent assay.

Results: F. nucleatum alone evoked a potent interleukin-8 response, whereas *S. cristatus* alone did not induce significant interleukin-8 expression in oral epithelial cells. When present together, *S. cristatus* attenuated the *F. nucleatum*-induced interleukin-8 production in the four oral epithelial cell lines to varying degrees. The inhibitory effect of *S. cristatus* was independent of its viability and its co-aggregation with *F. nucleatum*, was not related to soluble bacterial products and appeared to require bacterial contact with epithelial cells. Similar effects were seen with several other species of oral streptococci.

Conclusion: Our data suggest that *S. cristatus* may exert immunomodulatory effects on the interleukin-8 response of oral epithelial cells to *F. nucleatum* challenge.

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While the composition of the oral soft tissue microbiota is less well understood than that of dental plaque, emerging evidence has shown that oral streptococci and putative periodontal pathogens such as *Fusobacterium nucleatum*, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* normally colonize multiple oral soft tissue sites (1–3) and even invade mucosal epithelial cells (4). Within human buccal cells, a polymicrobial intracellular flora has been shown to be dominated by streptococci (5). These observations raise questions regarding how intracellular polymicrobial infections occur and how host cells respond under those conditions.

Our laboratory has approached these questions by employing *F. nucleatum* and *Streptococcus cristatus* as model organisms for the simultaneous infection of epithelial cells. *F. nucleatum* is an invasive gram-negative fusiform anaerobe that is frequently associated with periodontal diseases (6). Based on its ability to co-aggregate with a wide range of other plaque microorganisms, *F. nucleatum* is proposed to play a crucial role in plaque development (7). *S. cristatus*, a noninvasive member of the mitis group of oral streptococci, co-aggregates strongly with *F. nucleatum* to form structures known as 'corncobs' (8). We have previously observed that *F. nucleatum* can transport *S. cristatus* into KB and TERT-2 epithelial cells through a combination of co-aggregation and invasion mechanisms (9). Because the concurrent exposure of mucosal surfaces to these two species may not be a rare event in the oral cavity, this mode of entry might explain some aspects of polymicrobial intracellular colonization of buccal epithelial cells. Moreover, the combined infection model also provides us with a useful tool for examining the epithelial response to polymicrobial infections.

A characteristic response of epithelial cells to bacterial stimuli is the synthesis and release of inflammatory mediators such as cytokines (10). Many oral bacteria are able to induce a wide array of pro-inflammatory cytokines, such as interleukin-1β, interleukin-8, interleukin-6 and tumor necrosis factor-α, from epithelial cells (11–13). The inflammatory process is critical to host defense, but overproduction of inflammatory cytokines can cause epithelial damage. Thus, it seems necessary to have regulatory mechanisms, controlling innate immunity, operating at the level of epithelial cells in order to limit infection as well as to maintain tolerance to resident organisms. We recently identified a substantial number of live buccal cells associated with live bacteria in healthy human subjects (14), suggesting that live epithelial cells might be tolerant of bacterial invasion. Emerging evidence has shown that commensal bacteria behave in a manner different from that exhibited by pathogenic species, in that they induce very low levels of pro-inflammatory cytokines (15). Some commensal gut bacteria have been shown to be capable of attenuating the acute inflammatory cytokine responses triggered in intestinal epithelial cells (16,17). These findings suggest that commensal bacteria might contribute to immunological homeostasis at epithelial surfaces. Whether similar mechanisms take place in the oral environment has not been elucidated.

In this study, we used our *F. nucleatum* plus *S. cristatus* infection model to test the hypothesis that an oral commensal species is able to modulate epithelial pro-inflammatory responses to a putative oral pathogen. We demonstrated that *F. nucleatum* or *S. cristatus* alone induced distinct interleukin-8 expression patterns in oral epithelial cells and that *S. cristatus* dampened the epithelial interleukin-8 response to *F. nucleatum*. Our data suggest that *S. cristatus* may exert immunomodulatory effects on oral epithelial cells in response to pro-inflammatory stimuli.

Material and methods

Bacterial strains and culture conditions

F. nucleatum strains used included ATCC 10953, its isogenic co-aggregation-deficient mutant isolate 21 (9), ATCC 25580 and ATCC 49256. The streptococcal strains used were *S. cristatus* ATCC 49999 and 51110, *S. gordonii* DL-1, *S. sanguis* SK36, *S. parasanguis* ATCC 15912, *S. intermedius* ATCC 27335, *S. mutans* ATCC 25175, *S. salivarius* ATCC 25975, *S. mitis* SK612, *S. sobrinus* ATCC 27352 and *S. oralis* SK621. Other species included *P. gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *A. actinomycetemcomitans* ATCC 29522-4 and Y4 and *Eikenella corrodens* ATCC 23834. All bacteria were cultured under anaerobic conditions ($N_2/H_2/CO_2$, 8:1:1) at 37°C. *F. nucleatum*, *P. gingivalis and P. intermedia* were cultured in Trypticase Soy Broth (BBL, Becton-Dickinson, Sparks, MD, USA) supplemented with 1 g/L of yeast extract, 5 mg/L of hemin and 1 mg/L of menadione. *E. corrodens* was grown in Brain–Heart infusion broth (BBL). Streptococci and *A. actinomycetemcomitans* were grown in Todd–Hewitt broth (BBL). Heat-killed *S. cristatus* were prepared by heating the bacteria at 80°C for 10 min. Methanol-fixed *S. cristatus* were prepared by fixing the bacteria in 99% methanol for 1 h at room temperature. Conditioned medium was prepared by incubating *S. cristatus* with epithelial cells for 2 h followed by filtration of the cell supernatants through a 0.25-μm filter. The absence of bacterial growth after the above treatment was confirmed by plating samples on blood agar plates followed by incubation under anaerobic conditions for 3–5 d at 37°C. For inhibition of bacterial co-aggregation, *F. nucleatum* was pre-incubated with 1 mM L-canavanine and anti-*F. nucleatum* serum for 15 min at room temperature (9).

Epithelial cell cultures

The immortalized normal oral epithelial cells OKF6/TERT-2 were obtained under a materials transfer agreement from Dr James Rheinwald (Brigham and Women's Hospital, Boston, MA, USA). Three oral carcinoma cell lines (KB, TR146 and SCC15) were kindly provided by Dr Mark Herzberg (University of Minnesota, Minneapolis, MN, USA). All these cell lines were kept in 75-cm^2 flasks (Corning, Corning, NY, USA) in a humidified atmosphere of 5% CO_2 at 37°C. KB cells were maintained in minimal essential medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. TR146 cells were grown in Ham's F12 (Invitrogen) containing 10% fetal bovine serum. OKF6/TERT-2 and SCC15 cells were cultured in keratinocyte serum-free medium (Invitrogen) supplemented with $CaCl_2$ (0.4 mM), bovine pituitary extract (25 mg/mL) and epidermal growth factor (0.2 ng/mL). Twenty-four hours prior to infections, cells were seeded into triplicate wells in duplicate cell culture plates (Corning).

Bacterial infection procedure

Overnight broth cultures of bacteria were harvested, washed twice with 1 × Dulbecco's phosphate-buffered saline and then resuspended in serum-free cell culture medium. Bacteria were added to cell monolayers at various multiplicities of infection and incubated for various periods of time at 37°C in 5% CO_2. After stimulation, cell supernatants were collected for cytokine assays. Cells in one plate were used for RNA extraction (see below). For the other plate, an antibiotic protection assay was performed to assess intracellular invasion (9). In the Transwell system, streptococci were separated from KB cells and *F. nucleatum* by tissue culture inserts (pore size 0.45 μm) (Corning). After 2 h of incubation, cell monolayers were lysed and collected for RNA extraction. The viability of infected cells was examined by Trypan blue exclusion.

Enzyme-linked immunosorbent assay

The content of interleukin-8 in culture supernatants was quantified by enzyme-linked immunosorbent assay. Briefly, high-binding enzyme immunoassay plates (Costar) were coated with polyclonal antibodies to interleukin-8 (Pierce, Rockford, IL, USA) and blocked with phosphate-buffered saline containing 4% bovine serum albumin and 5% sucrose. Samples or a recombinant interleukin-8 standard (Pierce) were added to the wells for 1.5 h and then the plates were washed three times with phosphate-buffered saline containing 0.5% Tween 20. Biotinylated polyclonal antibodies to interleukin-8 (Pierce) were added and incubation was continued for a further 1.5 h. After extensive washing, NeutrAvidin-AP (Pierce) was added and the plates were washed again before the substrate p-nitrophenyl phosphate (PNPP) (Pierce) was added. When color developed, the reaction was stopped with 2 N NaOH and the absorbance was read at 405 nm in an automated enzyme-linked immunosorbent assay plate reader. A standard curve of absorbance vs. concentration of interleukin-8 was generated to determine the concentrations of interleukin-8 in samples. The detection limit of the assay was 10 pg/mL.

RNA extraction and reverse transcription–polymerase chain reaction

Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Three micrograms of RNA was reverse transcribed into cDNA that was then amplified by polymerase chain reaction using oligonucleotide primers specific for interleukin-8 or glyceraldehyde-3-phosphate dehydrogenase. The primer sequences used were as follows: interleukin-8, (forward) 5′-GAG ACA GCA GAG CAC ACA AGC-3′ and (reverse) 5′-TTC TCA GCC CTC TTC AAA AAC T-3′(375 bp); and glyceraldehyde-3-phosphate dehydrogenase, (forward) 5′-GAC CCC TTC ATT GAC CTC AAC TAC-3′ and (reverse) 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′ (220 bp). Thermal cycler parameters were optimized for each individual cell line, resulting in the use of different cycle numbers for different cell lines. Polymerase chain reaction without a cDNA template was performed as a negative control. Amplicons were visualized in 1.5% agarose gels stained by ethidium bromide.

Statistical analysis

Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test. Values that were statistically different are indicated by asterisks in the figures. Error bars indicate the mean ± standard deviation of three independent experiments performed in triplicate.

Results

S. cristatus attenuates *F. nucleatum*-induced interleukin-8 secretion by oral epithelial cells

To examine the effects of *S. cristatus* and/or *F. nucleatum* stimulation on epithelial interleukin-8 production, KB cells were incubated with *S. cristatus* 49999 and *F. nucleatum* 10953, either alone or in combination, at a multiplicity of infection of 100 over a time course from 0.5 to 6 h. Consistent with previous reports (12), exposure to *F. nucleatum* induced a significant increase in interleukin-8 production as early as 30 min (Fig. 1A). By contrast, no significant interleukin-8 secretion was noted following 6 h of infection with *S. cristatus* alone (Fig. 1A). Interestingly, induction of interleukin-8 by *F. nucleatum* was significantly attenuated by the presence of *S. cristatus* (Fig. 1A). The attenuating effect was observed first at 30 min and showed a pattern of increase with time (Fig. 1A). A 2-h incubation time was chosen in all subsequent experiments. The attenuating effect exerted by *S. cristatus* seemed quite strong, as a significant inhibition of interleukin-8 secretion was still noted at an *F. nucleatum* to *S. cristatus* ratio of 10:1 (Fig. 1B). For optimal results, a 1:1 ratio was used in all the following experiments.

To verify that the attenuating effect on interleukin-8 production was not limited to the KB cell line, we next examined the interleukin-8 responses in three additional oral epithelial cell lines: TERT2, TR146 and SCC15. Although they all showed statistically significant interleukin-8 attenuation at the protein level following co-incubation with *F. nucleatum and S. cristatus* (Fig. 2B), the four cell lines had a variable magnitude of response to bacterial challenge, with the most pronounced effect seen in KB and TR146 cells and the lowest effect in TERT2 cells. For convenience, KB cells were used as a model to study the epithelial cytokine responses in most of the following experiments.

To rule out the possibility that cell toxicity caused by *S. cristatus* infection might have been responsible for the interleukin-8 attenuation, the viability of the cells was evaluated by Trypan blue exclusion. No obvious cytotoxic effects were detected at either multiplicity of infection tested within a 6-h incubation time for all the cell lines tested, and cell viability was ≥ 95% of the untreated control in all experiments. Thus, under our assay conditions, epithelial cell integrity was not compromised.

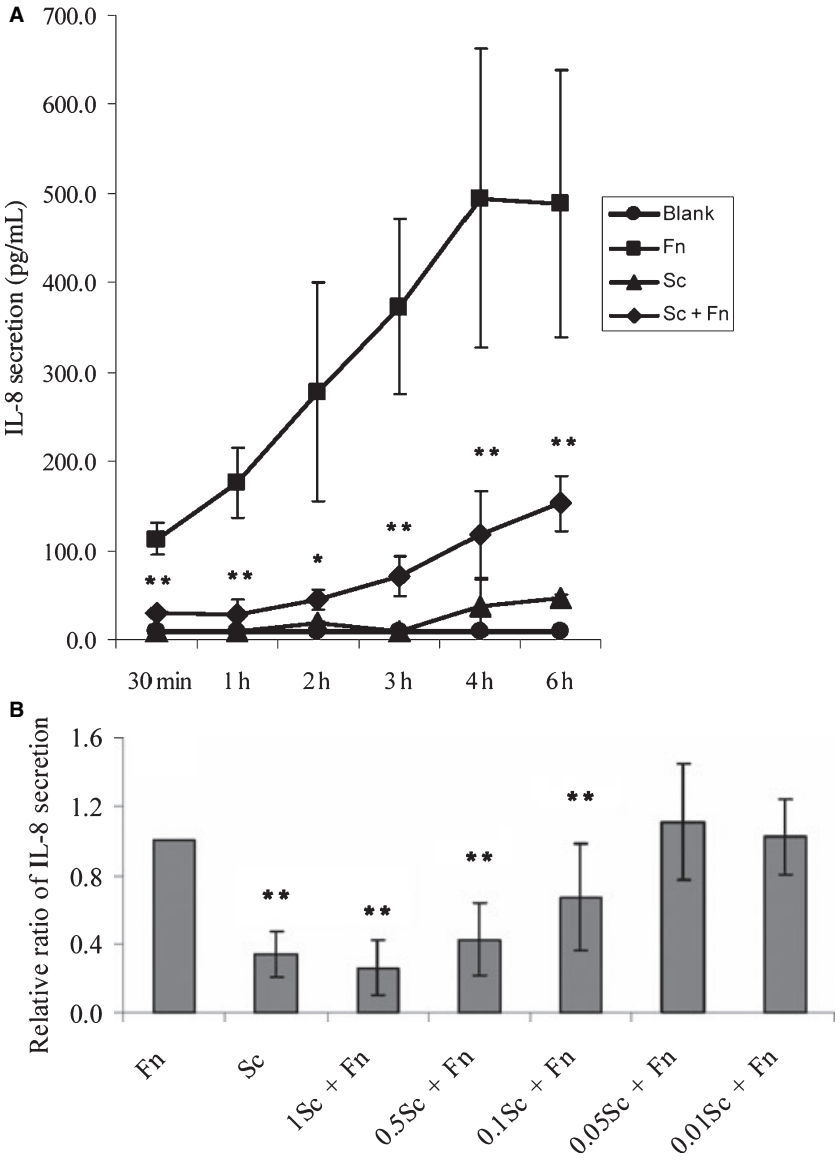

Fig. 1. The inhibitory effect of *Streptococcus cristatus* on *Fusobacterium nucleatum*-induced interleukin-8 production in KB cells. (A) *S. cristatus* inhibited *F. nucleatum*-induced up-regulation of interleukin-8 in KB cells over a 6-h time-period. KB monolayers were treated with bacteria at a multiplicity of infection of 100, or with medium alone, for various periods of time. (B) Dose–response of *S. cristatus* on *F. nucleatum*-induced interleukin-8 production. *F. nucleatum* was used at a multiplicity of infection of 100. *S. cristatus* was used at different multiplicities of infection, from 1 to 100 (which are relatively expressed as 0.01, 0.05, 0.1, 0.5, or 1 *S cristatus* per *F. nucleatum*). Interleukin-8 levels were measured by enzyme-linked immunosorbent assay. The results are expressed as means ± standard deviation from three independent experiments. **, $p < 0.01$; *, $p < 0.05$ compared with *F. nucleatum* alone. Blank, medium control; Fn, *F. nucleatum*; IL-8, interlukin-8; Sc, *S. cristatus*.

Streptococcal presence, but not viability, is required for interleukin-8 attenuation

To determine the effect of streptococcal viability on interleukin-8 attenuation, heat-killed and methanol-fixed *S. cristatus* were used to infect KB cells. Killing was confirmed by culture on agar plates. Surprisingly, we found that dead *S. cristatus* were still able to inhibit *F. nucleatum*-induced interleukin-8 production to a similar extent as live bacteria (Fig. 3A,B). However, neither *S. cristatus*-conditioned medium (Fig. 3A,B) nor live *S. cristatus* separated from epithelial cells and *F. nucleatum* by a tissue culture insert (Fig. 3C) exerted any attenuating effect. Moreover, the interleukin-8 content was not reduced in cell supernatants that were first collected from *F. nucleatum*-infected TERT2 cells and then incubated with *S. cristatus* (Fig. 3D). All of these results suggested that the observed interleukin-8 attenuation seemed not to be mediated by secreted proteases of *S. cristatus*. It appeared that either direct host cell–streptococcal cell contact, or interaction between the two species, was necessary for interleukin-8 inhibition.

Co-aggregation does not affect the attenuating effect of *S. cristatus*

It has been shown that *F. nucleatum* promotes the internalization of *S. cristatus* into epithelial cells via a combination of co-aggregation and invasion mechanisms (9). In the present study, various approaches were employed to investigate the role of co-aggregation in interleukin-8 attenuation induced by *S. cristatus*.

First, anti-*F. nucleatum* serum was used to pretreat *F. nucleatum* for 30 min, which has been shown to inhibit interbacterial co-aggregation by ≈50% (9). Antibody-treated *F. nucleatum* induced significantly less interleukin-8 secretion than untreated bacteria, but interleukin-8 levels were still significantly higher than those produced by dual infection (Fig. 4).

Second, 1 mM L-canavanine (an analogue of L-arginine that inhibits co-aggregation by ≈80%) (9) was used to pretreat *F. nucleatum*. In a similar manner to the results for pretreatment with anti-*F. nucleatum* serum, interleukin-8 induction by L-canavanine-treated *F. nucleatum* in the presence of *S. cristatus* was significantly reduced when compared with pretreated *F. nucleatum* alone, although pretreated *F. nucleatum* induced lower interleukin-8 secretion than untreated control bacteria (Fig. 4).

Lastly, we employed a co-aggregation-deficient spontaneous mutant of *F. nucleatum* (Isolate 21) in our assay. The interleukin-8 response to Isolate

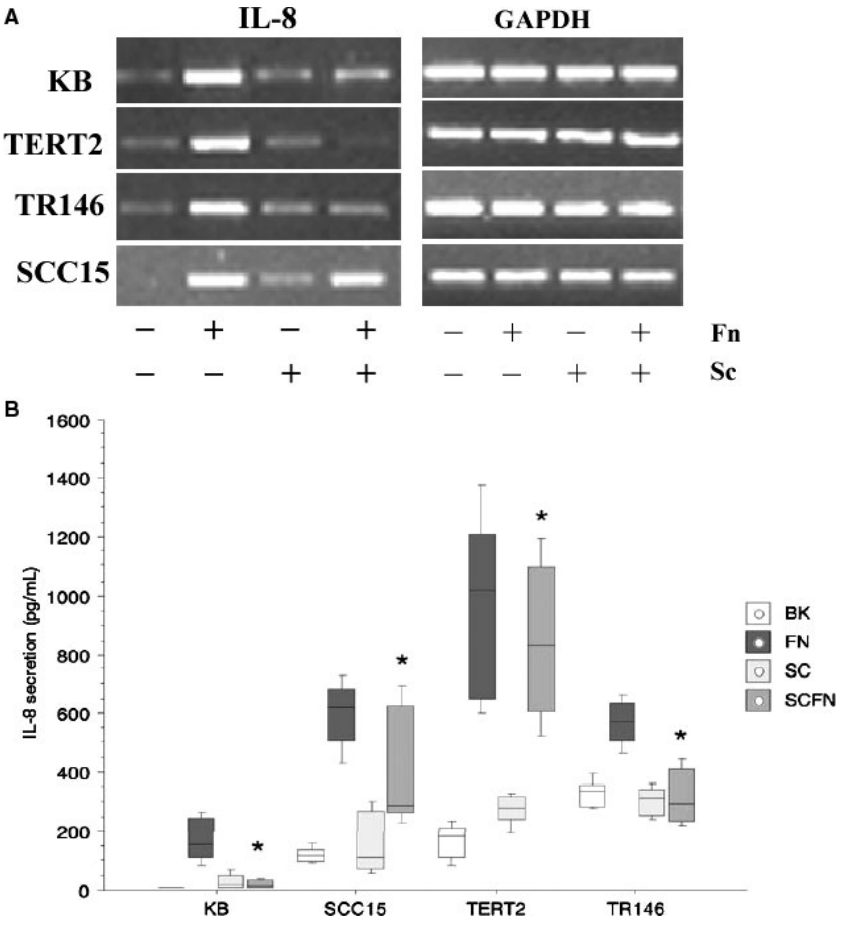

Fig. 2. The attenuating effect on interleukin-8 production induced by *Streptococcus cristatus* in four oral epithelial cell lines. KB, TERT2, TR146 and SCC15 cell monolayers were treated with either bacteria or medium alone for 2 h at a multiplicity of infection of 100. Total mRNA was extracted and reverse transcription-polymerase chain reaction was performed using primers for interleukin-8 and glyceraldehyde-3-phosphate dehydrogenase (A). Glyceraldehyde-3-phosphate dehydrogenase mRNA expression was used as a positive and a loading control. Fn, *F. nucleatum*; Sc, *S. cristatus*. Culture supernatants were harvested for interleukin-8 measurement, which was carried out by enzyme-linked immunosorbent assay (B). The results are displayed as box-and-whisker plots. Median values are denoted by the horizontal line across each box. The upper and lower bounds of the boxes represent the 75th and 25th percentiles; the upper and lower whiskers represent the 95th and 5th percentiles. The asterisks denote differences between *F. nucleatum* alone and *F. nucleatum* plus *S. cristatus* that were statistically significant at alpha = 0.05. The actual *p*-values for each cell line were: KB, $p = 0.0004835$; SCC15, $p = 0.0000135$; TERT2, $p = 0.0059415$, and TR146, $p = 0.0000025$. BK, medium control; FN, *F. nucleatum*; SC, *S. cristatus*; SCFN, *S. cristatus* plus *F. nucleatum*.

21 alone was not significantly different from the response to wild-type *F. nucleatum* alone, and its induction of interleukin-8 was also significantly inhibited by the presence of *S. cristatus* (Fig. 4).

Taken together, the disruption of co-aggregation did not appear to affect *S. cristatus*-induced interleukin-8 attenuation, whereas L-canavanine and anti-*F. nucleatum* serum alone showed a weak inhibitory effect.

S. cristatus inhibits epithelial interleukin-8 release induced by various oral bacteria

To investigate whether the attenuating effect of *S. cristatus* was specific to the fusobacterial strain used, we studied interleukin-8 secretion in KB cells induced by other fusobacterial strains and other oral species, including *F. nucleatum* 25580 and 49256, *A. actinomycetemcomitans*, *E. corrodens*, *P. gingivalis* and *P. intermedia*. The two fusobacterial strains alone induced significantly higher interleukin-8 production, and that response was significantly attenuated in the presence of *S. cristatus*. A similar effect was also noted for *E. corrodens* (Fig. 5). Four *A. actinomycetemcomitans* strains did not induce detectable interleukin-8 protein induction in KB cells except in the presence of *S. cristatus*. However, the levels observed were not significantly different from those seen with *S. cristatus* alone (Fig. 5). Neither *P. gingivalis* nor *P. intermedia* induced detectable interleukin-8, either alone or in combination with *S. cristatus* (data not shown).

The attenuating effect on interleukin-8 may be shared by other streptococcal species

S. cristatus is a member of the mitis group of oral viridans streptococci (18). To determine whether the attenuating effect of *S. cristatus* was strain specific and/or species specific, *S. cristatus* ATCC 51100 and nine related oral streptococcal species were co-incubated with *F. nucleatum* to stimulate KB cells. With a 2-h incubation period at a multiplicity of infection of 100, *S. gordonii*, *S. sanguis*, *S. parasanguis*, *S. mutans*, *S. mitis* and *S. oralis* attenuated interleukin-8 secretion to the baseline level (data not shown). However, there were a few exceptions. There was no significant difference between *F. nucleatum* alone and *S. sobrinus* plus *F. nucleatum*. By contrast, *S. intermedius* plus *F. nucleatum*, and *S. salivarius* plus *F. nucleatum* both induced a significantly higher interleukin-8 response compared with *F. nucleatum* alone (Fig. 6).

Discussion

The synthesis and release of cytokines by epithelial cells in response to microbial challenge is considered to be an early stage of inflammation (10).

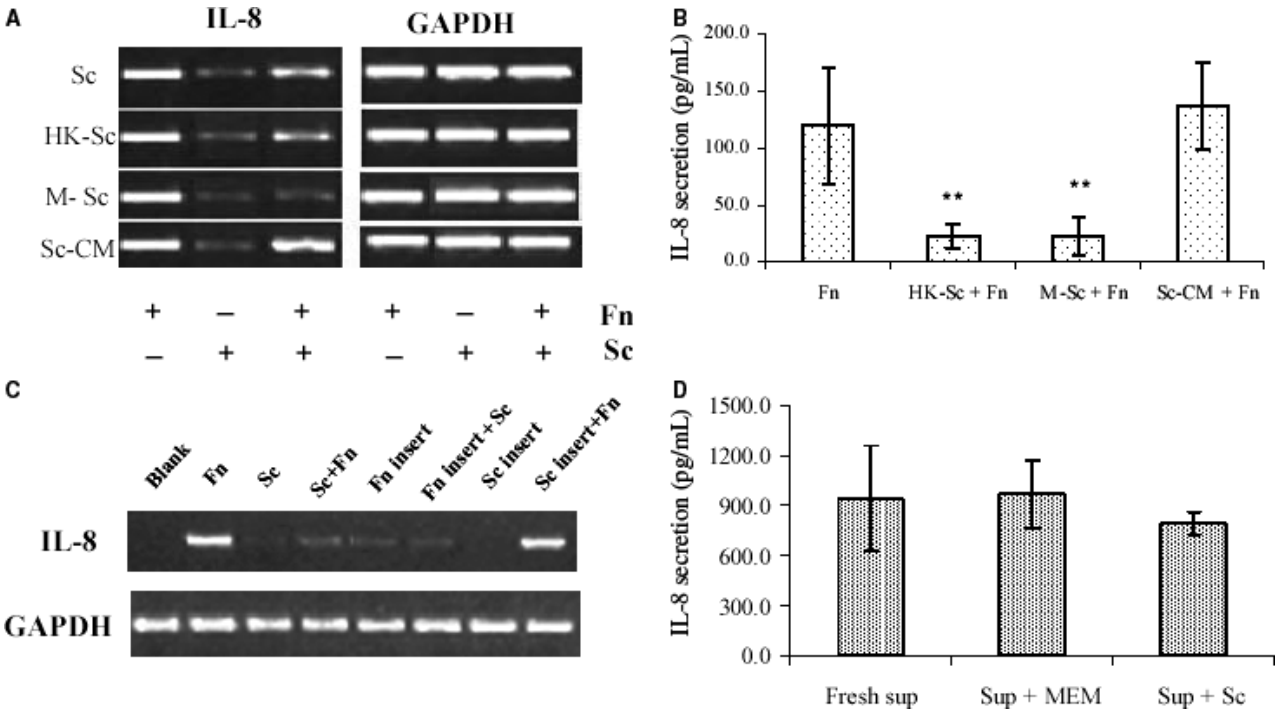

Fig. 3. The effects of various *Streptococcus cristatus* products on *Fusobacterium nucleatum*-induced interleukin-8 production. KB cell monolayers were cocultured with live *S. cristatus*, heat-killed *S. cristatus*, methanol-fixed *S. cristatus*, or *S. cristatus*-conditioned cell culture medium for 2 h. Interleukin-8 mRNA and protein were assayed by reverse transcription-polymerase chain reaction (A) and enzyme-linked immunosorbent assay (B), respectively. For protease activity assay, first, *S. cristatus* was separated from KB cells and *F. nucleatum* through a tissue culture insert (pore size 0.45 μm) in the coculture system (Sc insert + Fn). After 2 h of incubation, interleukin-8 mRNA was assayed by reverse transcription-polymerase chain reaction (C). Second, filtered *F. nucleatum*-conditioned cell culture medium was incubated either with *S. cristatus* or with minimal essential medium alone for 2 h and the interleukin-8 level was then measured by enzyme-linked immunosorbent assay (D). Values represent means ± standard deviation of three independent assays. **, $p < 0.01$, compared with *F. nucleatum* alone. Fn, *Fusobacterium nucleatum*; Fn insert, *F. nucleatum* separated from KB cells through a tissue insert; Fn insert + Sc, *F. nucleatum* separated from KB cells through a tissue insert plus *S. cristatus*; Fresh sup, filtered *F. nucleatum*-conditioned cell culture medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HK-Sc, Heat-killed *S. cristatus*; HK-Sc + Fn, Heat-killed *S. cristatus* plus *F. nucleatum*; IL-8, interleukin-8; M-Sc, Methanol-fixed *S. cristatus*; M-Sc + Fn, Methanol-fixed *S. cristatus* plus *F. nucleatum*; Sc, *Streptococcus cristatus*; Sc-CM, *S. cristatus*-conditioned cell culture medium; Sc-CM + Fn, *S. cristatus*-conditioned cell culture medium plus *F. nucleatum*; Sc + Fn, *S. cristatus* plus *F. nucleatum*; Sc insert, *S. cristatus* separated from KB cells through a tissue culture insert; Sc insert + Fn, *S. cristatus* separated from KB cells through a tissue culture insert plus *F. nucleatum*; Sup + MEM, filtered *F. nucleatum*-conditioned medium co-incubated with minimal essential medium; Sup + Sc, filtered *F. nucleatum*-conditioned cell culture medium co-incubated with *S. cristatus*.

Both commensal and pathogenic microorganisms have molecular motifs that can activate pro-inflammatory gene expression, but generally the exposed epithelium can maintain an immune homeostasis in response to its established microbial flora. Diverse mechanisms have been proposed that involve crosstalk between bacteria and epithelial and lamina-propria cells to quench inflammation (19). It is a relatively new idea that commensals may also actively contribute to this process. In this report we showed that *S. cristatus*, a commensal oral viridans streptococcus, is able to attenuate the interleukin-8 response to *F. nucleatum*, a putative oral pathogen, in oral epithelial cells. This attenuating effect required contact of *S. cristatus* with host cells, but seemed to be independent of streptococcal viability and interbacterial co-aggregation. Moreover, this effect appeared to be shared by other commensal streptococci. These data support the hypothesis that certain oral commensal streptococci may attenuate epithelial pro-inflammatory responses to potential pathogens.

Interleukin-8 is a key host response molecule in the protection against microbial infections because it is able to activate neutrophils and monocytes (20). Deregulated production of interleukin-8 has been related to the pathogenesis of inflammatory bowel disease and chronic adult periodontitis (21). In the present study, we observed that epithelial exposure to *S. cristatus* greatly decreased interleukin-8 levels induced by *F. nucleatum* alone. *P. gingivalis* has been reported to inhibit interleukin-8 release strongly from gingival epithelial cells, which was partially ascribed to the action of its extracellular proteases (22). Protease activity seemed not to be a factor in our study, because *S. cristatus* neither degraded pre-existing interleukin-8 in *F. nucleatum*-stimulated cell culture media nor secreted protease to degrade

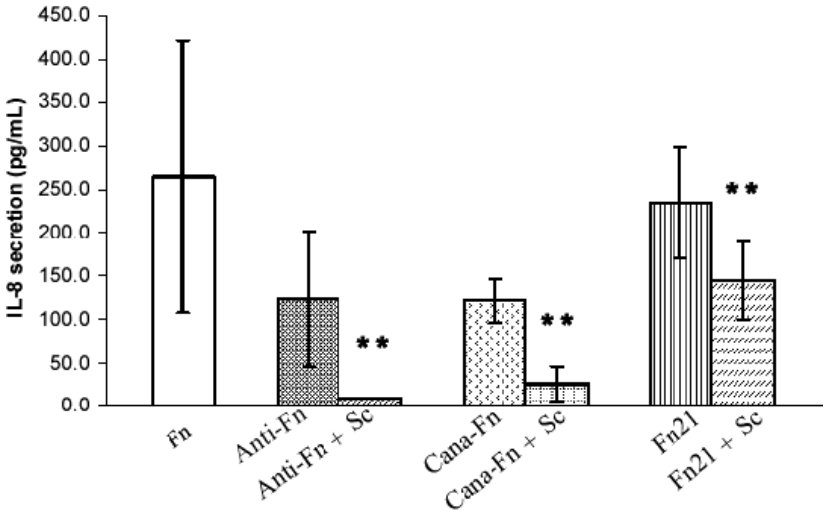

Fig. 4. The effect of co-aggregation on *Fusobacterium nucleatum*-induced interleukin-8 production. Anti-*F. nucleatum* serum or L-canavanine was used to disrupt interspecies co-aggregation. Isolate 21, a co-aggregation-defective *F. nucleatum* isolate was also employed. The pretreated *F. nucleatum* or Fn21 were used to stimulate KB cells, either alone or in combination with *S. cristatus*. The interleukin-8 levels in cell supernatants were assayed using enzyme-linked immunosorbent assay. Results are expressed as means ± standard deviation from three independent experiments. **, $p < 0.01$; *, $p < 0.05$; compared with treated *F. nucleatum* alone. Cana-Fn, L-canavinine pre-treated *F. nucleatum*; Cana-Fn + Sc, L-canavinine pre-treated *F. nucleatum* plus *S. cristatus*; Fn, *Fusobacterium nucleatum*; *Fn21*, the designated name for a co-aggregation defective *F. nucleatum* isolate; Fn21 + Sc, *Fn21* and *S. cristatus*; IL-8, interleukin-8; Sc, *Streptococcus cristatus*.

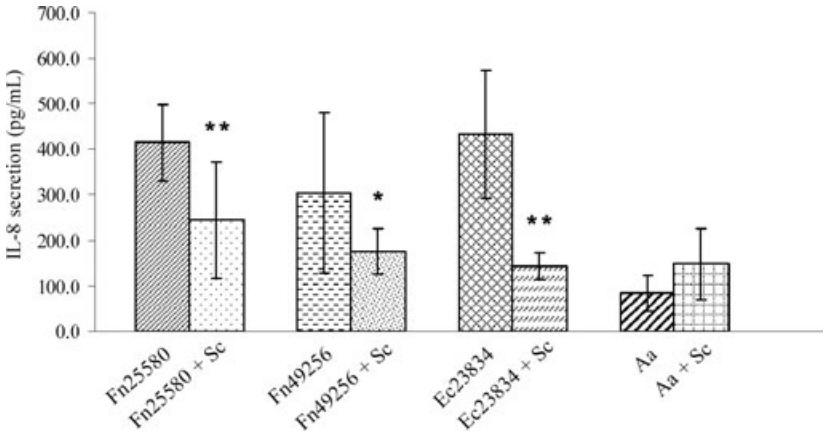

Fig. 5. The effect of *Streptococcus cristatus* on interleukin-8 production induced by different species. Two strains of *Fusobacterium nucleatum* (Fn25580 and Fn49256), and one strain each of *Eikenella corrodens* (Ec23834) and *Actinobacillus actinomycetemcomitans* (Aa), were used to stimulate the KB cells, alone or in combination with *S. cristatus*. The interleukin-8 levels in cell supernatants were assayed by enzyme-linked immunosorbent assay. Results are expressed as means ± standard deviation from three independent experiments. **, $p < 0.01$; *, $p < 0.05$; compared with the corresponding stimulus alone. IL-8, interleukin-8; Sc, *Streptococcus cristatus*.

the interleukin-8 induced by *F. nucleatum* in a Transwell culture system. Certain commensal and probiotic bacteria have been shown to inhibit interleukin-8 synthesis induced by tumor necrosis factor-α and pathogenic species in human intestinal epithelial cell lines, but only when they were alive (16,17,23). By contrast, our results showed that *S. cristatus* did not have to be alive to induce inhibitory effects. Similarly, Zhang *et al*. reported that living, heat-killed and antibiotic-treated versions of the probiotic strain *Lactobacillus rhamnosus* GG all diminished the tumor necrosis factor-α-induced interleukin-8 response in Caco-2 cells (24). It has been reported that CpG DNA mediates the anti-inflammatory effect of an γ-irradiated probiotic product through Toll-like receptor 9 signaling in a murine model of colitis (25). Because the heat-killing method used in our study denatured bacterial DNA, the mechanism for the inhibitory effect of heat-killed streptococci may be independent of the Toll-like receptor 9 pathway. In support of this, our recent studies found no visible attenuation of *F. nucleatum* by streptococcal genomic DNA (preliminary data, not shown).

In our model system, cell-to-cell contact can occur at two levels: between bacteria and epithelial cells; and between bacterial species. We recently demonstrated that interbacterial co-aggregation is responsible for the enhanced adhesion and invasion of oral epithelial cells by *S. cristatus* in the presence of *F. nucleatum* (9). Because such interactions might affect the ability of *F. nucleatum* to recognize or activate epithelial cell-surface receptors, it is possible that the attenuating effect of *S. cristatus* is related to its co-aggregation with *F. nucleatum*. However, several lines of evidence do not support this speculation: first, both epithelial attachment and invasion by *F. nucleatum* were not significantly affected in the presence of *S. cristatus* (9); second, disruption of interbacterial co-aggregation by various means did not affect the attenuating effect of *S. cristatus*; and, third, potent interleukin-8 production induced by a co-aggregation-defective fusobacterial isolate (that displays similar levels of adhesion and internalization to wild-type *F. nucleatum*) was efficiently inhibited by *S. cristatus*. These results suggest that the attenuating effect of *S. cristatus* is not likely to occur

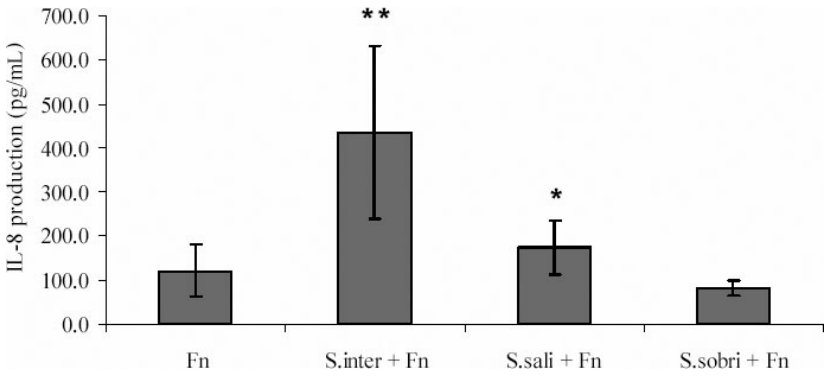

Fig. 6. The effects of various oral streptococci on *Fusobacterium nucleatum*-induced interleukin-8 production. A different *Streptococcus cristatus* strain (ATCC 51100) and nine related oral streptococcal species were used, either alone or in combination with *F. nucleatum*, to infect the KB cells. The interleukin-8 levels in cell supernatants were assayed by enzyme-linked immunosorbent assay. Data shown here are only for *F. nucleatum* alone (Fn), *F. nucleatum* with *S. intermedius* (S.inter + Fn), *F. nucleatum* with *S. salivarius* (S.sali + Fn), and *F. nucleatum* with *S. sobrinus* (S.sobri + Fn). Results are expressed as means ± standard deviation from three independent experiments. **, $p < 0.01$; *, $p < 0.05$; compared with *F. nucleatum* alone. IL-8, interleukin-8.

through interfering with the interaction between *F. nucleatum* and host epithelial cells.

Another level at which *S. cristatus* might exert its modulating effect is through interaction with host epithelial cells. Indeed, this mode of action has been supported by numerous studies of gut commensal or probiotic bacteria (16,17,23,24). The results of two experiments in our study suggested that direct contact between streptococci and oral epithelial cells is necessary for the anti-inflammatory effect: first, *S. cristatus*-conditioned medium containing no bacteria showed no inhibitory effect on *F. nucleatum*-induced interleukin-8 production; and, second, separation of *S. cristatus* from epithelial cells by tissue culture inserts abolished the attenuating effect on interleukin-8 production induced by *F. nucleatum* situated in the lower chamber of the culture system.

The exact mechanism(s) of the oral streptococci-induced anti-inflammatory response could not be ascertained from our current results. It is known that oral epithelial cells constitutively express various pattern recognition receptors, including Toll-like receptors (26). Binding of microbial ligands to Toll-like receptors largely results in the production of pro-inflammatory cytokines, including interleukin-8, through activation of the nuclear factor-κB pathway. That response is strictly regulated to ensure the appropriate modulation of both immune and inflammatory responses and to avoid unintended stimulation. Known mechanisms act at multiple levels of the Toll-like receptor signaling cascade, including the toll-interacting protein, TOLLIP (27), single immunoglobin interleukin-1R-related molecule (28) and cytoplasmic peptidoglycan receptor NOD2 protein (29). Recently, probiotics and commensals have been recognized to suppress pathogen-induced pro-inflammatory responses in intestinal models, therefore helping to maintain immune homeostasis (16,17). Often, the inhibitory effect of a probiotic or commensal organism is ascribed to blockade of nuclear factor-κB activation, either by inducing nuclear clearance through up-regulated peroxisome proliferation-activated receptor gamma (16), or by preventing nuclear translocation of the active subunit through inhibition of nuclear factor κB inhibitor α (IκB-α) degradation (17). It appears that mechanisms vary between different strains and species of bacteria, and between different models. However, all cases, including ours, have reported that bacterial–epithelial contact is required, suggesting that receptor systems antagonistic to Toll-like receptor action are involved. Further studies to determine the underlying molecular mechanisms for *S. cristatus*-mediated attenuation are currently in progress.

It is an inevitable problem in tissue culture models that different cell lines vary in biological activities. In the present study we tested four commonly used oral epithelial cell lines, including an immortalized normal cell line and carcinoma cells from three different sources. Our results revealed that those cell lines did vary in the magnitude of their interleukin-8 response to bacterial infection. It does not appear that this was a result of differences between normal cells and cancer cells, because the three cancer cell lines also varied among themselves. Despite these variations, all cell lines were consistent in being able to mount an interleukin-8 response to the challenge of *F. nucleatum*, which could be significantly attenuated by *S. cristatus*. A recent study, using gingival epithelial cells as a model, also indicated that certain oral streptococcal species may have an anti-inflammatory effect (30), which suggests that our conclusions from the present study may be applicable to a broad range of cell types. While we can never rule out the possibility that another cell line might behave differently, our findings do suggest that *S. cristatus* is a generally effective modulator of the epithelial pro-inflammatory response to *F. nucleatum*. In addition, the presence of *S. cristatus* also inhibited the interleukin-8 production induced by *E. corrodens*, indicating that the anti-inflammatory effect of *S. cristatus* might work for different stimuli. Interestingly, we found that the attenuating effect of *S. cristatus* may extend to other oral viridians streptococci, including *S. mitis and S. oralis*, which are prominent soft tissue colonizers (3). In the oral cavity, a diverse intracellular flora dominated by streptococci is often identified in healthy human buccal epithelial cells (5). That flora also includes a variety of putative periodontal pathogens, including *F. nucleatum* (4,5). Inhibition of the acute inflammatory response, demonstrated by oral commensal streptococci

in the present study, might represent a mechanism that contributes to tolerance of pro-inflammatory species on and in epithelial surfaces. This may be relevant to the natural history of periodontal disease, because extracrevicular epithelial cells may act as a reservoir from which pathogens can recolonize the gingival crevice following periodontal treatment (4,5, unpublished data, J. Johnson and J. Rudney).

In summary, we demonstrated that *S. cristatus* and certain other streptococcal species have the capability to dampen the interleukin-8 response in different oral epithelial cell lines. Determining the molecular mechanism of action by these organisms may provide new insights into immune homeostasis of the oral epithelium and lead to the development of new therapeutics for mucosal inflammatory diseases.

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